SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Clinical course, prognosis and phenotypes in mixed connective tissue disease

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The Examination takes place at Division of Clinical Immunology, Faculty of Medicine, University of Debrecen,
11:00 a.m. 19. February, 2014.

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The PhD Defense takes place at the Lecture Hall of Department of Dermatology, Faculty of Medicine, University of Debrecen
14:00 p.m. 19. February, 2014.

INTRODUCTION

In the early 1970's a new disease was added to the family of connective tissue diseases (CTDs), denoted as Mixed Connective Tissue Disease (MCTD). These patients showed a number of shared clinical features including Raynaud's phenomenon, arthritis, puffy hands, abnormal esophageal motility, lymphadenopathy and myositis. Sharp et al. described MCTD as an "apparently distinct rheumatic disease syndrome associated with high titer of antibody to an extractable nuclear antigen".

During the next 20 years many studies were published reporting contradictory views about the nature of MCTD, and the original description required considerable modifications. Organ involvement is often more extensive than was previously thought; neurological entity and pulmonary disease, erosive arthritis, various skin signs, and vasculitis has been added to the clinical picture. Very little is known about the mortality, morbidity, and factors, influencing the disease outcome in MCTD. Earlier reports showed that the leading cause of death in MCTD is pulmonary arterial hypertension (PAH) with obliterative vasculopathy. The high number of deaths in MCTD is also associated with involvement of internal organs related to the disease, such as lung, cerebrovascular and renal disease.

AIMS

1. Our aim was to analyze the survival and causes of death of 280 patients with MCTD who were followed up at our division during a 30-years' period. Our further aim was to search for prognostic factors, and find clinical features and autoantibodies associated with mortality in MCTD.

2. The phenotypes may vary in patients with MCTD, and the clinical signs and disease course can be modified by the presence of different autoantibodies. We intended to assess and categorize the phenotypes of MCTD patients. We catalogued symptoms, which were characteristic to distinct MCTD subsets, and besides anti-U1RNP, other autoantibodies, which can affect the clinical features were investigated. Furthermore we attempted to identify, whether the subdivision of MCTD into distinct major groups had any prognostic consequences.

3. Further aim was to evaluate the vitamin D status in patients with MCTD and to determine which clinical symptoms, laboratory parameters and endothelial cell markers are associated with low vitamin D levels.

4. We assessed the immune mediated sensorineural hearing loss in patients with MCTD. Our aim was to investigate which clinical signs, autoantibodies, and immunological makers influence the development of immune mediated sensorineural hearing loss in MCTD.

5. Further aim of the present study was to assess B cell subsets in MCTD in the active and inactive stage of the disease, compared to healthy individuals. In order to evaluate the clinical relevance of these findings, the frequencies of B cell subsets were correlated with disease activity and autoantibody levels.

PATIENTS AND METHODS

Patients

We recruited 280 consecutive patients with MCTD who were followed up at the Division of Clinical Immunology, University of Debrecen between 1979 and 2011.

The classification criteria for MCTD were used described "by Alarcon-Segovia et al." including Raynaud's phenomenon, swelling of the hands with a spindle-like swelling of the fingers, sclerodactyly, synovitis, myositis, and the presence of anti-U1-RNP autoantibodies in the patients' sera.

The first investigations were made at time of diagnosis of MCTD, the patients were followed up every three or four months. Diagnostic procedures for MCTD included X-ray, CT, HRCT and MRI imaging, lung function tests, electromyography, electroneurography, radionuclid esophageal transit scintigraphy, radiographic passage, nailfold capillary microscopy.

During the follow-up period the biopsies were carried out at University of Debrecen, Department of Surgery, the histological evaluation was done at the Department of Pathology.

Muscle biopsy was performed in 105 patients, skin biopsy in 47 patients, open lung biopsy in 6 cases, transbronchial biopsy in 14 cases, renal biopsy in 8 patients, thyroid gland biopsy in 52 patients and liver biopsy in 3 patients.

PAH was diagnosed by Doppler echocardiography, when the systolic pressure of the right ventricle was ≥25 Hgmm at rest. Right ventricle catheterization was performed on 22 patients with simultaneous Doppler echocardiography.

In MCTD patients secondary antiphospholipid syndrome (APS) was defined if at least one of the 2 major clinical criteria (vascular thrombosis, pregnancy morbidity) and one of the laboratory criteria were met. Laboratory criteria include aCL antibody of IgG and/or IgM isotype, anti-β2-GPI antibody IgG and/or IgM isotype or lupus anticoagulant (LA) present in plasma on two or more occasions at least 12 weeks apart.

Clinical disease activity was assessed by the systemic lupus activity measure (SLAM) retrospectively from the patients' reports. A SLAM value >6 was considered high disease activity.

Laboratory and immunological studies

All patients underwent a full blood count, kidney and liver function tests, creatine phosphokinase (CK), and urine-analysis.

Antinuclear antibodies (ANA) were detected on human epithelial cell line 2 substrate (HEp-2 cells) by indirect immunofluorescence. ANA was considered positive, when sera titers were above 1: 64.

Serum concentrations of **autoantibodies** were analyzed by commercial enzyme-linked immunosorbent assays (ELISA; anti-U1-RNP: Pharmacia and Upjohn, Freiburg, Germany; anti-SSA, anti-SSB, anti-Jo1, anti-Scl70: Hycor Biomedical, Indianapolis, USA; anti-double stranded (ds) DNA, anti-cardiolipin (anti-CL IgG, IgM, IgA), anti-ß2-glycoprotein I (anti-ß2-GPI IgG, IgM, IgA): Orgentec, Mainz, Germany; anti-cyclic citrullinated peptide (anti-CCP): Immunoscan CCPlus, Euro-Diagnostica, Malmö, Sweden).

Plasma levels of **anti-endothelial cell autoantibodies** (AECA) were determined by a cellular ELISA with cultured human umbilical vein endothelial cells.

Plasma **cytokine levels** were measured by ELISA following the manufacturer's instructions (IL-23 ELISA kits, from Bender MedSystems, Burlingame, CA, USA; IL-10, IL-17, IL-6, IFN-γ and IL-12 (p40) ELISA kits R&D system, Minneapolis, MN, USA).

Thrombomodulin (TM) (Diagnostic Stago, Asnieres, France) and **endothelin-1 (ET-1)** (Biomedica medizinprodukte Gesellschaft mbH and o KG, Vienna) levels were measured by ELISA using commercial reagents according to the manufacturer's instructions. **Von Willebrand factor antigen (VWFAg)** was measured by an immunoturbidimetric assay using STA Liatest VWF from Diagnostica Stago.

Peripheral B cell analysis:

10-20 ml heparinized freshly-drawn (<2 hours) whole blood was obtained from patients and controls. Peripheral blood mononuclear cells (PBMCs) were separated immediately by density gradient centrifugation over Ficoll-Hypaque (Sigma-Aldrich). Immunofluorescent staining was performed by incubating PBMCs with the following monoclonal antibodies at optimal concentration: peridinin chlorophyll protein-Cy5.5 (PerCP-Cy5.5)-labeled anti-

CD19 (BioLegend, San Diego, CA, USA); allo-phycocyanin (APC)-labeled anti-CD27; phycoerythrin (PE)-labeled anti-IgD; fluorescein isothiocyanate (FITC)-labeled anti-CD38; FITC-labeled anti-CD95 (BD Biosciences, Heidelberg, Germany). Stained cells were washed and fixed in 1% paraformaldehyde, and data were collected by performing four-color flow cytometry using FACSCalibur cytometer (BD Biosciences). Data were analyzed using CellQuest software (BD).

For **CD4+CD25^{high}FoxP3+ regulative T cell analysis** PBMCs were separated by density gradient centrifugation over Ficoll-Hypaque. For immunofluorescent staining anti-CD4-FITC and anti-CD25-PC5 anti-human monoclonal antibodies were used. The intracellular FoxP3 staining was performed according to the manufacturer's instruction. Data were collected by performing four-color flow cytometry using FACSCalibur cytometer. Data were analyzed with CellQuest software.

The absolute numbers of the distinct B- and T-cell subsets were calculated by multiplying the relative proportion of a particular B- or T-cell population with the absolute number of lymphocytes determined by routine laboratory tests on the same day.

Intracytoplasmic IL-10 and IFN-γ cytokine assessment of CD4+ T-cells:
Lymphocytes were stimulated with phorbol-miristate-acetate (PMA, 25 ng/ml) and Ionomycin (1 μg/ml), the surface CD4 staining was performed with anti-human CD4-PC5 antibodies. After using FACS Permeabilizing Solution the intracellular staining of CD4+ T-cell subsets was done with the following monoclonal antibodies: FITC-labeled anti-IFN-γ, PE-conjugated anti-IL-10 (BD Biosciences). Measurement was done with Beckman Coulter FC500 flow cytometry (Beckman Coulter Inc., Miami, FL).

25(OH)D vitamin levels were determined at the Department of Clinical Biochemistry and Molecular Pathology Laboratory of the University of Debrecen. Samples were taken during summer period (from June to October), and analyzed by HPLC using a Jasco HPLC system (Jasco, Tokyo, Japan) and Bio-

Rad reagent kit (Bio-Rad Laboratories, Hercules, CA, USA). Vitamin D deficiency has been defined as a 25(OH)D <20 ng/ml; vitamin D insufficiency as 21-29 ng/ml and Vitamin D sufficiency as >30 ng/ml.

Statistical analysis

For survival analysis data were analyzed using SPSS for Windows 20.0 statistic software. Differences in frequencies of systemic manifestations among the living and deceased patients and correlation among the supposed prognostic factors were examined using the chi^2 test. The univariate analysis of the survival was performed with Kaplan-Meier method. The log rank test was used to determine the statistical significance of the observed differences in the survival rates between the different organ involvements.

Non-hierarchical K-means cluster analysis (Stata Package, StataCorp. 2007, Stata Statistical software: Release 10. College Station TX: Stata Corp LP) was performed to identify groups of MCTD with similar clinical and autoantibody patterns.

For clustering the MCTD patients we evaluated the following clinical symptoms and autoantibodies in each patient: Raynaud's phenomenon, PAH, myositis, ILD, erosive arthritis, AECA and anti-CCP antibodies. Three clusters of MCTD patients were defined based on their very different clinical and immunoserological parameters. Comparison between the 3 clusters was carried out using one-way analysis of variance (ANOVA) for continuous variables and conventional chi-square test for proportions.

The association between vitamin D serum concentrations and disease activity, autoantibodies, biological markers and cardiovascular risk factors in MCTD patients had been investigated by Pearson correlation. These relationships had been evaluated in multivariate model as well (controlling for confounding effect of age, BMI, family history of cardiovascular diseases, smoking, diabetes mellitus, menopause and serum glucose concentration) by linear regression analysis. Both unstandardized and standardized coefficients had been calculated. The connection between the presence of clinical symptoms in MCTD patients and their vitamin D serum concentrations had been described by univariate and

multivariate logistic regression. Later models were controlled for the same confounding factors as the linear regression analyses.
P values less than or equal to 0.05 were considered statistically significant.

RESULTS

1. Long-term follow-up of patients with MCTD

Clinical features

We recruited 280 consecutive patients with MCTD who were followed up at the Division of Clinical Immunology, University of Debrecen between 1979 and 2011. The mean±SD age of the total 280 patients (259 female and 21 male) at the time of the investigation was 53.1±12.6 years (range: 19-78 yrs). The mean follow-up of the disease was 13.1±7.5 years (range: 1-33 yrs).

During the follow-up period the most frequent symptoms in our series were polyarthritis (89.6 %), Raynaud's phenomenon (57.5 %), interstitial lung disease (ILD) (50.3 %), esophageal dysmotility (49.6 %), and sclerodactyly (41.8 %). The frequency of esophageal hypomotility, nervous system disease increased, and new symptoms developed, such as pulmonary arterial hypertension, interstitial lung disease, thrombotic events, and renal disease.

One hundred and thirty two patients had interstitial lung disease (ILD). ILD was recurrent in 24 cases. Pulmonary biopsy was obtained in 16 cases, when we could not exclude tumor, sarcoidosis or lymphoma. The histological investigation in all 16 patients showed nonspecific interstitial pneumonitis (NSIP) with mononuclear cell infiltration in the lung parenchyma. Immunohistochemical analysis in all cases revealed epithelial deposits of C3 complement and immunoglobulin M. Interestingly, no patients' sera with ILD were positive for anti-Jo1 antibody.

Pulmonary arterial hypertension (PAH) developed in 50 patients 14.5±3.71 years after the diagnosis of MCTD. In our series the pulmonary arterial pressure was between 40-60 mmHg measured by echocardiography, confirmed by right heart pulmonary arterial catheterization. All patients with PAH had continuously high levels of anti-U1RNP (>30 U/ml), while 42/50 patients (84.0 %) were positive to AECA. These findings suggest that high levels of anti-U1RNP can contribute to endothelial cell proliferation.

In our series renal involvement was observed only in 3,9 %. Three patients had thrombotic thrombocytopenic purpura or hemolytic uremic syndrome (TTP/HUS) associated nephropathy. Eight patients had glomerulonephritis (GN). Every 8 patients with

proteinuria underwent renal biopsy, and the biopsies showed ISN class II mesangial proliferative glomerulonephritis in 5 patients, and focal nephritis in 3 patients.

Cardiovascular disease such as cardiomyopathy, valvular changes, arrhythmia, and ischaemic heart disease was found in 98 patients.

Seventy-two patients had thrombotic events with antiphospholipid antibody positivity, and these patients fulfilled the classification criteria for APS. Fifty-nine of 72 (81.9%) patients had venous thromboses, 9 (12.5%) patients suffered from cerebral vascular attacks, and 4 (5.5 %) had arterial occlusion in the lower extremities.

Malignancy developed in 16 MCTD patients during the disease course: gastrointestinal cancer was the most common malignancy (n = 8) followed by breast (n=2), cervix cancer (n = 2), bronchial cancer (n = 2), pancreatic and esophageal cancer (1-1 for each type). Amongst those 258 patients who survived, 10 patients had tumor (3.8 %), while among the 22 deceased patients 6 patients (27.2%) suffered from cancer, however; the cause of death was not the tumor itself.

Autoantibodies in MCTD

Antinuclear antibodies and anti-U1RNP autoantibodies were detected in all patients' sera. However, patients' sera were positive for other autoantibodies, such as AECA (33.5%), anti-CL IgG, IgA, and IgM (35.0 %), (IgG n=58, IgA n=21, IgM n=19 patients), anti-β2-GPI (28.2%) (IgG n=40, IgA n=22, IgM n=17 patients), anti-CCP autoantibodies (18.9 %), as well as anti-SSA (32.8 %), while anti-ds-DNA (3.2 %) and anti-Sm (6.7 %) antibodies were also present.

We found a close association between cardiovascular events and the presence of anti-CL IgG, IgM and IgA autoantibodies [IgG/IgM: RR: 2.925 (95% CI: 1.50-5.7); IgA: RR: 3.059, (95% CI: 1.15-8.1]. We found that anti-β2-GPI IgG, IgM and IgA antibodies were more frequent in patients with cardiovascular diseases [anti-β2-GPI IgG/ IgM (RR: 2.79, 95% CI: 1.41-5.5), anti-β2-GPI IgA: RR: 6.2 (95 % CI: 2.2-17.7)].

We found association between arterial/venous thrombotic events and AECA (RR: 2.917 95% CI: 1.58-5.36), and anti-CL IgG antibodies (RR: 31.39, 95% CI: 1.76-54.26).

Patients with PAH showed association with AECA (RR: 56.43, 95% CI: 19.8-160.8) and anti-CL (RR: 3.67, 95 % CI: 1.61-8.3).

Causes of death in MCTD

During the observational period, 22 of 280 patients died. PAH was the major complication of MCTD. During the follow-up period in spite of adequate therapy, 9 patients died 0.5-2 years after the diagnosis of PAH.

Three patients died in Thrombotic Thrombocytopenic Purpura/ Hemolytic Uremic Syndrome (TTP/HUS). The occurrence of TTP in MCTD is rare. In one patient fresh cytomegalovirus (CMV) infection provoked TTP. The patients with TTP did not respond to steroid pulse therapy, plasma exchange or fresh frozen plasma, and finally died 4-8 days after the onset of neuropsychiatric symptoms. These patients suffered from end-stage renal failure and the prognosis was extremely poor.

Three MCTD patients died due to infections. Two patients had hepatitis C infection, which provoked hepatic coma after 12 and 15 years of diagnosis of MCTD. One patient died of Staphylococcal sepsis.

Cardiovascular events in MCTD were associated with a relatively poor prognosis. In the deceased patient group 2 patients had dilatative cardiomyopathy (DCM), and congestive heart failure caused the death, accompanied by ventricular arrhythmias. The underlying pathophysiologic mechanisms that caused DCM could be myocarditis, coronary artery disease, and small vessel vasculopathy of the myocardium. In one patient the autopsy showed severe myocarditis. Four patients had coronary sclerosis with ischaemic cardiomyopathy.

The 5, 10 and 15 year survival rates were 98%, 96% and 88 % for patients with MCTD.

We used univariate analysis to compare the clinical parameters and the presence of autoantibodies between the living and deceased patient. The deceased patients were younger, compared

to those who survived (deceased vs. living patients: 49.3±15.2 vs. 54.0±12.3, p=0.03; respectively), while there was no difference between the follow-up periods (deceased and living patients: 13.2±7.6 vs. 12.3±7.5; p=0.835).

Our cohort study showed that the presence of PAH (p=0.0071, RR: 3.664, 95% CI:.47-9.133), esophageal hypomotility (p=0.04, RR: 2.837, 95% CI: 1.076-7.483), serositis (p<0.001, RR:4.79, 95% CI: 1.926-11.927) and kidney involvement (p=0.04, RR:4.93, 95% CI: 1.208-20.147) increased the risk of the mortality. Cardiovascular events (p<0.001, RR: 3.625, 95% CI:.463-8.979), secondary APS (p=0.039, RR:2.63, 95% CI: 1.085-6.394) and malignant tumors (p<0.001, RR: 9.3, 95% CI: 2.99-28-837) also negatively influenced the MCTD patients' survival.

The presence of anti-CL (p=0.019, RR: 2.94, 95% CI: 1.209-7.151), anti-β2GPI (p=0.002, RR: 4.202, 95% CI: 1.717-10.283) and AECA (p=0.002, RR: 6.154, 95% CI: 2.30-16.321) autoantibodies increased the risk of mortality.

The high-dose corticosteroid or immunosuppressive therapy did not influence the patients' survival.

2. Phenotypes in MCTD

The classification criteria for MCTD require the presence of anti-U1RNP autoantibodies; however AECA, anti-cardiolipin (anti-CL) and anti-CCP antibodies can modify the clinical symptoms and disease course so the phenotypes may vary in patients with MCTD.

We applied multivariate analysis to investigate and characterize different subsets of MCTD patients. We catalogued symptoms, which were characteristic to distinct MCTD subsets, and besides anti-U1RNP, other autoantibodies, which can affect the clinical features were investigated.

Three subgroups of 201 patients were defined, based on their distinct clinical and immunoserological parameters. According to the classification criteria, all patients were positive to anti-U1-RNP. Cluster 1 included 77 patients, cluster 2: 79 patients, while cluster 3: 45 patients with MCTD.

The mean age of MCTD patients was 52.9±12.4 years (range: 31-88 years). The patients in cluster 1 were significant

younger at the time of the investigation compared to clusters 2 and 3 (50.8±12.7 vs. 55.1±9.7 and 56.1±14.7 years, $p<0.001$). The mean follow-up of MCTD was 12.5±7.2 years (range: 1-30 years), and was similar in all clusters (15.5±11.4 vs. 14.2±8.2 vs. 14.5±8.0 years, n.s.).

The prevalence of the organ involvement and autoantibody titers was statistically different among clusters.

The prevalence of PAH (55.8 %; $p<0.001$), livedo reticularis (24.6 %, $p< 0.001$), and Raynaud's phenomenon (92.2 %; $p<0.001$) was significant higher in cluster 1 than in cluster 2 and 3. In cluster 1 a close association was found between PAH and AECA ($p< 0.001$, OD: 24.618, 5.206-116.42), also between PAH and anti-CL antibodies ($p<0.001$, OD: 12.0, 3.823-37.669). Cluster 1 was characterized by the highest incidence of secondary antiphospholipid syndrome (50.6 %, $p<0.001$).

In cluster 2, the incidence of ILD (98.7 %; $p<0.001$), myositis (77.2 %; $p< 0.001$), and esophageal dysmotility (89.8 %; $p<0.001$) was significant greater than that in cluster 1 and 3.

In cluster 3 42/44 (93.3 %) MCTD patients had erosive arthritis, and 88.0% of these patients' sera contained anti-CCP antibodies besides anti-U1RNP. The bone erosions are located mainly in the wrists, interphalangeal and metacarpal-phalangeal joints of the hands, and less frequently in the feet. Interestingly erosions developed 5 and 10 years after the onset of MCTD. Patients with anti-CCP positivity also had myositis, sclerodactyly, serositis, ILD but the frequency of these symptoms was less than in cluster 1 or 2.

Patients in cluster 1 had predominantly vascular damages, such as PAH, Raynaud's phenomenon with several capillary damages. Cluster 2 MCTD patients had ILD associated with myositis, while patients in cluster 3 had frank arthritis.

During the follow-up, PAH was the major factor contributing to severe illness and/or death in our patients. Cluster 1 had significantly worse cumulative survival than cluster 2 or 3.

3. Immune mediated sensorineural hearing loss in MCTD

Audio-vestibular dysfunction is not unusual in autoimmune diseases. Sensorineural hearing loss (SNHL), which may occur as a distinct entity involving exclusively the inner ear, is more commonly part of a systemic autoimmune disorder. Contrary to other systemic autoimmune diseases, the association between MCTD and audio-vestibular impairment has not been described.

The study population consisted of 71 patients (2 men and 69 women) with MCTD and 51 age- and sex-matched healthy control individuals (2 men, 49 women). All patients were regularly followed up at the Autoimmune Outpatient Clinic of the 3rd Department of Medicine, Medical and Health Science Center, University of Debrecen between January 2006 and July 2006. The patients' mean age was 57.1±7.9 years and a mean disease duration of MCTD was 14.5±8.0 years. All patients and controls underwent audiological evaluation that included pure tone and speech audiometry. In addition, the systemic manifestations of the disease and drug therapy were recorded.

Thirty-three (46.47%) of 71 patients with MCTD were proved to have SNHL by audiogram, versus only 11 (21.5%) controls ($p < 0.007$).

An association was found between Raynaud's phenomenon, secondary APS, and SNHL [Raynaud's phenomenon: $p < 0.03$; RR: 3.125 (1.128–8.659); secondary APS: $p < 0.05$; RR: 3.3 (1.007–10.816)].

We found a close association between the serum levels of anti-U1RNP and SNHL [anti-RNP: $p < 0.05$; RR: 2.683 (1.025–7.026)]. We identified a correlation between the presence of AECA and IgG type aCL antibodies and SNHL [AECA: $p < 0.0001$; RR: 8.750 (2.986–25.639); aCL IgG: $p < 0.0001$; RR: 36.96 (9.694–140.982)]. In MCTD patients with SNHL, significantly higher levels of anti-U1RNP autoantibodies were found compared with the normal hearing MCTD group (MCTD+SNHL: 19.3 ± 10.2 U/ml; MCTD–SNHL: 13.8 ± 11.3 U/ml; $p < 0.05$). Further, AECA levels were significantly elevated in MCTD patients with SNHL (MCTD+SNHL: 41.2 ± 31.8 U/ml; MCTD–SNHL: 23.9 ± 19.0 U/ml; $p < 0.001$).

Serum levels of IFN-gamma and TNF-alpha were increased in MCTD patients with SNHL compared to MCTD patients without SNHL (MCTD+SNHL: IFN-gamma: 51.8 ± 22.1 pg/ml, without SNHL: 39.5 ± 32.0 pg/ml, $p < 0.05$; TNF-alpha with SNHL: 34.7 ± 23.2 pg/ml, without SNHL: 22.1 ± 14.7 pg/ml, $p < 0.05$). MCTD patients with SNHL showed an increase in serum IL-10 levels compared to patients with intact hearing (MCTD+SNHL: 26.5 ± 10.4 pg/ml, MCTD patients without SNHL: 22.8 ± 17.8 pg/ml, $p < 0.05$), while serum IL-4 levels were similar in MCTD patients with SNHL than in controls.

The percentage and absolute number of CD4+CD25highFoxP3+ natural regulatory T cells (nTreg) were significantly lower in peripheral blood of patients with MCTD compared to controls. Interestingly, the percentage and the absolute number of CD4+CD25highnTregs showed a decrease in MCTD patients with SNHL compared to patients without impaired hearing (MCTD+SNHL: 2.01 ± 1.33% vs controls: 4.36 ±0.99%, $p < 0.001$; MCTD–SNHL: 3.14 ± 1.74% vs controls: $p < 0.05$; MCTD with and without SNHL: 2.01 ± 1.33% vs 3.14 ± 1.74% $p < 0.001$; absolute number: MCTD+SNHL: 0.028 ± 0.005 G/l, MCTD–SNHL: 0.011 ± 0.012 G/l, $p < 0.05$, controls: 0.04 ± 0.016 G/l, MCTD-SNHL vs controls: $p < 0.001$, MCTD–SNHL vs controls: $p < 0.001$).

In contrast, in MCTD patients with and without SNHL the percentage and absolute number of CD4+/IL-10+ Tregs significantly increased compared to controls. Although the percentage and absolute number of CD4+/IL-10+ cells were higher in MCTD patients with SNHL, the difference was not statistically significant.

4. Vitamin D deficiency and cardiovascular risk factors in MCTD

It is known that the deficiency of active vitamin D is associated with increased incidence and severity of several autoimmune diseases . In addition, vitamin D deficiency has been linked to cardiovascular risk factors, including hypertension,

diabetes and dyslipidemia, suggesting a possible relationship of low vitamin D levels and cardiovascular morbidity and mortality.

We evaluated the vitamin D status in patients with MCTD and determined which clinical symptoms, laboratory parameters and endothelial cell markers are associated with low vitamin D levels.

125 female MCTD patients and 48 age- and sex-matched healthy controls were enrolled in the study.

The clinical symptoms, autoantibodies (anti-U1-RNP, anti-cardiolipin — anti-CL and anti-endothelial cell antibody — AECA), serum cytokines (IFN-γ, IL-6, IL-12, IL-23, IL-17 and IL-10), soluble endothelial cell markers (endothelin, thrombomodulin — TM, and von Willebrand factor antigen — vWFAg) and serum lipids (total cholesterol, triglyceride, LDL-C, HDL-C, apolipoprotein A1, and apolipoprotein B) were investigated for an association with vitamin D levels by univariate and multivariate statistical analyses.

In the MCTD patients' group the age and disease duration were 53.65 years (range: 22–79 years) and 12.96 years (range: 2–35 years) respectively.

The mean vitamin D level for MCTD patients was significantly lower compared to the age- and sex-matched control group (26.16±13.5 ng/ml vs. 34.92±9.64 ng/ml; pb0.001).

A negative association was found between vitamin D serum concentrations and disease activity (p=0.001) in MCTD patients with multivariate linear regression analysis.

The serum levels of anti-U1-RNP (p=0.022) and anti-CL IgA (p=0.015) antibodies were higher in MCTD and significantly associated with hypovitaminosis D according to Pearson correlation analysis. Anti-CL IgG and AECA levels were also higher in MCTD patients than in controls, although the association with vitamin D deficiency was not significant (p=0.154 and p=0.117).

T helper 1 cytokines (IL-12 and IFN-γ) and T helper 17 cytokine (IL-17) levels were higher in patients than in the healthy controls, but vitamin D status did not influence their plasma levels. However the IL-23 showed a negative correlation with the vitamin D level (p=0.011), and the elevated levels of serum IL-6 (p<0.001) and IL-10 (p=0.033) were also significantly associated with lower vitamin D levels. TM (p=0.001) and ET-1 (p=0.033) levels, and the carotid intima media thickness (p<0.001) were found to have a significant inverse association with the serum vitamin D level.

Regarding traditional CV risk factors, we found a significant inverse correlation between vitamin D level and serum total cholesterol (p=0.042), ApoA1 (p=0.004) and homocysteine (p=0.046) according to a multivariate linear regression analysis controlled for age, BMI, family history of cardiovascular diseases, smoking, diabetes mellitus, menopause and serum glucose. Serum fibrinogen (p=0.010) was also inversely associated with vitamin D levels.

Systolic/diastolic blood pressure, triglyceride, LDL-C, HDL-C, ApoB, PON1 activity and hs-CRP levels did not depend on vitamin D level in MCTD patients.

We examined the association of clinical symptoms of MCTD patients with serum 25(OH)D levels. The univariate analysis showed, that lower vitamin D levels were significantly associatedwith pulmonary arterial hypertension (PAH) (p=0.038), secondary antiphospholipid syndrome (p=0.045) and cardiovascular disease (p=0.002). After adjustment for age, BMI, family history of cardiovascular diseases, smoking, diabetes mellitus, menopause and serum glucose, the inverse association between vitamin D level and cardiovasculardisease still remained significant (p<0.001).

5. Derailed B cell homeostasis in MCTD

Humoral autoimmune processes, the presence of autoantibodies is a central feature of MCTD. Hypergammaglobulinemia and polyclonal B cell hyperactivity are characteristic features in MCTD patients. Although the exact pathogenesis of MCTD is still unknown, B cell abnormalities characterized by autoantibody formation and polyclonal B cell activation play an important role.

Peripheral blood samples were obtained from 46 MCTD patients (all women). The mean±SD age of the MCTD patients was 53.7±10.6 years (r: 37–70 years) and the duration of their disease was 10.1±6.2 years (r: 3–29 years). Twenty age- and sex-matched healthy individuals served as controls (mean age: 53.9±10.2, r: 38–67 years). Disease activity, severity of MCTD patients was assessed by the systemic lupus activity measure (SLAM). SLAM value >6 was considered high disease activity. The mean activity score was

6.56±3.66 (r: 2–13). Twenty-seven out of 46 patients with MCTD exhibited a flare at the time of analysis.

Peripheral blood B cells, including plasma cells, can be identified, as CD19+ cells. CD19+ cells can be distributed into different B cell-subsets according to their additional expression of CD27, IgD and CD38.

The data showed several alterations in the distribution of B cell subsets in patients with MCTD, including increased CD27-IgD + CD38low naive B cells, increased CD19 + CD27-IgD + CD38high transitional B cells, CD27-IgD- (double negative B cells) and CD27high plasma cells. The frequencies of non-switched (CD27 + IgD+) and switched (CD27 + IgD-) memory B cells were similar to the healthy controls.

The earliest B cell stage, which can be detected in the peripheral circulation, has been termed ‘‘transitional B cells’’. These cells express CD38high and characterized by the absence of surface CD27 expression. Active, untreated MCTD patients had both elevated percentages and absolute numbers of CD19 + CD27-IgD + CD38high transitional B cells, compared to the inactive stage and controls (MCTD active and inactive stage: 6.1 (4.2–11.3)% vs. 3.2 (2.4–5.4)%, $p < 0.009$; absolute number: 13,9 (8–30) cells/µl vs. 9 (3–14) cells/µl, respectively, $p < 0.045$).

Both the relative and absolute number of naive B cells (CD27-IgD + CD38low) was higher in the active disease, than in the inactive stage (MCTD active and inactive stage 72.0 (62.1–88.3)% vs. 55.3(45.3–70.2)% $p < 0.002$; 116 (98–145) cells/µl vs. 45 (29–86) cells/µl, respectively, $p < 0.001$). The distribution of CD19 + CD27+ B cells in patients with MCTD was similar to controls (CD27 + MCTD and controls: 30.83% (16.36–43.47) vs. 31.63% (23.0–39.5, $p = 0.504$). Both percentage and absolute number of the switched memory B cell population (CD27 + IgD-) was similar in the active and inactive stage (MCTD active and inactive stage: 11.18 (6.80–15.70)% vs. 21.2 (9.22–26.63)%, ns; absolute number: 12 (6–38) cells/µl vs. 14 (11–38 cells/µl, ns).

Plasma cells uniquely express a very high density of CD27, while memory B cells are CD27 positive with a lower density, therefore B cells expressing CD27high permits a good analysis to identify Ig producing B cells. The distribution of CD27high plasma cells was significant higher in patients with MCTD compared to

healthy controls, while in the active stage, the distribution of CD27high plasma cells further increased (CD27high: MCTD active and inactive stage: 1.66 (0.850–2.44)% vs. 0.67 (0.49–0.96)%, respectively, $p < 0.001$). A close correlation was observed between the frequency of CD27high plasma cells and anti-U1RNP concentration in the patients' sera.

Recently a new population of memory B cells was reported, containing isotype-switched (IgG and IgA) and IgM- only cells, lacking the expression of CD27 and IgD surface markers, called double negative (DN) B cells. Jacobi et al. previously described that DN B cells can bear CD95 surface marker. The number of the double negative B cells (CD27-IgD-) increased in MCTD patients compared to healthy individuals, and the percentage was higher in the active stage (DN B cells: MCTD active and inactive stage: 7.8 (4.14–10.9)% vs. 3.6 (1.88–5.43)%, respectively, $p < 0.001$). The frequency of CD95+ expression of CD27-IgD- B cells was significantly higher in active MCTD patients than in controls (CD95 expression of CD27-IgD- B cells: MCTD and controls: 31.13 (19.45–42.81)% vs. 21.60 (16.80–24.50)%, respectively, $p < 0.001$). There was a close correlation between the number of DN CD95+ B cells and disease activity ($r = 0.51$; $p < 0.001$).

CONCLUSIONS

- MCTD is a distinct entity, with well-defined clinical symptoms. The probability of survival of patients is better than 20 years ago. Despite the modern treatment, pulmonary arterial hypertension remained the leading cause of death in MCTD. The prevalence of cardiovascular morbidity and mortality, malignancy, and thrombotic events increased during the disease course of MCTD. Pathological inflammatory mechanisms are clearly present in MCTD that can lead to impaired endothelial function. The presence of antiphospholipid antibodies also raised the risk of mortality. Concerning aggressive therapeutic regimes in MCTD, they decrease organ damage, yet may cause novel complications such as infections, accelerated atherosclerosis, or cancer.

- Based on our cluster analysis, we assume that the assessment of the autoantibody profile and accurate cataloging of clinical characteristics in MCTD can help to identify prognostic factors and can be indicative of a disease flare and have a pivotal role in mortality and survival assessment. Our cluster analysis may help to understand the spectrum of the clinical symptoms of MCTD, and aid in understanding the role of various autoantibodies in the development of organ involvement in MCTD. We believe that these analyses also aid the early start of appropriate therapy in these patients before serious irreversible organ damage appears and can therefore lead to a better quality of life and better disease outcome in MCTD.

- Sensorinerual hearing loss in patients with MCTD has not previously been described. We found significantly higher prevalence of SNHL in MCTD patients compared to controls. In MCTD, SNHL is a specific organ manifestation and appears frequently. We have found that pathogenic autoantibodies, decreased levels of regulatory T cells, and overexpression of proinflammatory cytokines may play a role in the pathogenesis of immune mediated inner ear disorders in MCTD.

- Vitamin D insufficiency is present in 59.2% of patients with MCTD.

Low levels of vitamin D are associated with the presence of antiphospholipid antibodies and secondary antiphospholipid syndrome in MCTD. Low levels of vitamin D show a close correlation to inflammatory cytokines (IFN-γ, IL-23, IL-6 and IL-17) and disease activity of MCTD. Vitamin D insufficiency is also correlated with endothelial cell dysfunction and lipid abnormalities.

Vitamin D deficiency in MCTD is associated with the inflammatory process and traditional risk factors which can explain the high prevalence of cardiovascular disease in MCTD.

- Our study is the first to assess the peripheral blood B cell subsets in patients with MCTD. Our results indicated disturbed homeostasis of peripheral B cells in MCTD.

B cell abnormalities in MCTD are characterized by elevated numbers of transitional and naive B lymphocytes and the expansion of CD27-IgD-CD95+ B memory cells showed a close correlation with disease activity. The number of CD27high plasma cells increased and we could identify an association between the number of CD27high plasma cells and the level of anti-U1-RNP autoantibodies. These findings may help to monitor diseases activity and humoral autoimmune processes in patients with MCTD.

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List of publications related to the dissertation

1. **Hajas, Á.**, Szodoray, P., Nakken, B., Gaál, J., Zöld, É., Laczik, R., Demeter, N., Nagy, G., Szekanecz, Z., Zeher, M., Szegedi, G., Bodolay, E.: Clinical course, prognosis, and causes of death in mixed connective tissue disease.
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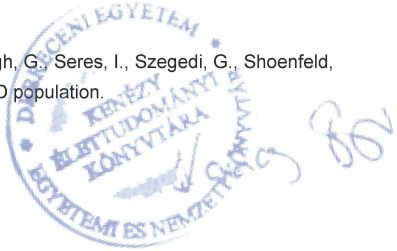

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6. Zöld, É., Szodoray, P., Nakken, B., Baráth, S., Kappelmayer, J., Csáthy, L., **Hajas, Á.**, Sipka, S., Gyimesi, E., Gaál, J., Barta, Z., Hallay, J., Szegedi, G., Bodolay, E.: Alfacalcidol treatment restores derailed immune-regulation in patients with undifferentiated connective tissue disease.
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